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14. ABSTRACT
We proposed to test a hypothesis that cell fusion between tumor cells and between tumor and normal cells contributes to metastasis. This contribution can be implemented by two mechanisms, by generating cells with diverse genetic and epigenetic properties, and by providing tumor cells with qualities of normal cells that are required to reside in normal tissues. This hypothesis might explain why cells tumor cells can grow at distant sites, why they express proteins that are normally expressed by cells of the metastasized tissue, and why only a minute fraction of cells released by the primary tumors form metastases. The funded research focuses on two specific aims, to determine the mechanism of gene transfer between prostate cancer cells (Aim 1); and to determine whether cell fusion affects metastatic properties of prostate cancer cells (Aim 2). During this reporting period, we identified the mechanism of gene transfer, thus completing Aim 1. The unexpected finding that the transfer was carried out by a virus prompted us to initiate a new line of research the first step of which will be to identify the virus. This finding also prompted us to develop a new approach for cell fusion, which will serve as the main technique for the experiments proposed in Aim 2. Accomplishing this Aim will be the main focus of our research for the remaining funding period.

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Prostate Cancer, cell fusion, metastasis

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INTRODUCTION: The main goal of the research funded by this grant is to test the hypothesis that fusion among tumor cells or fusion of tumor to normal cells facilitates metastasis. The initial observation that led to the proposed research was the finding that human prostate cancer cells PC3 that were transduced either with green fluorescent protein EGFP ("green" PC3 cells) or red fluorescent protein RFP ("red" PC# cells) injected into mice produced tumors composed of cells that expressed both protein ("yellow" cells). The "yellow" cells had enhanced metastatic potential, which suggested that the horizontal exchange of the genetic information affected cell malignancy. We proposed to identify the mechanism of genetic exchange (Aim 1), with the main hypothesis being that the gene exchange was mediated by cell fusion, and to test whether cell fusion caused by viruses can affect ability of PC3 cells to metastasize (Aim 2).

KEY RESEARCH ACCOMPLISHMENTS: AIM 1. To determine the mechanism of gene transfer between prostate cancer cells. We proposed three hypotheses to explain how the genes were transferred. Our favorite was that the transfer is a result of cell fusion, while the second was the engulfment of apoptotic bodies, a previously described mechanism of horizontal gene transfer. The third

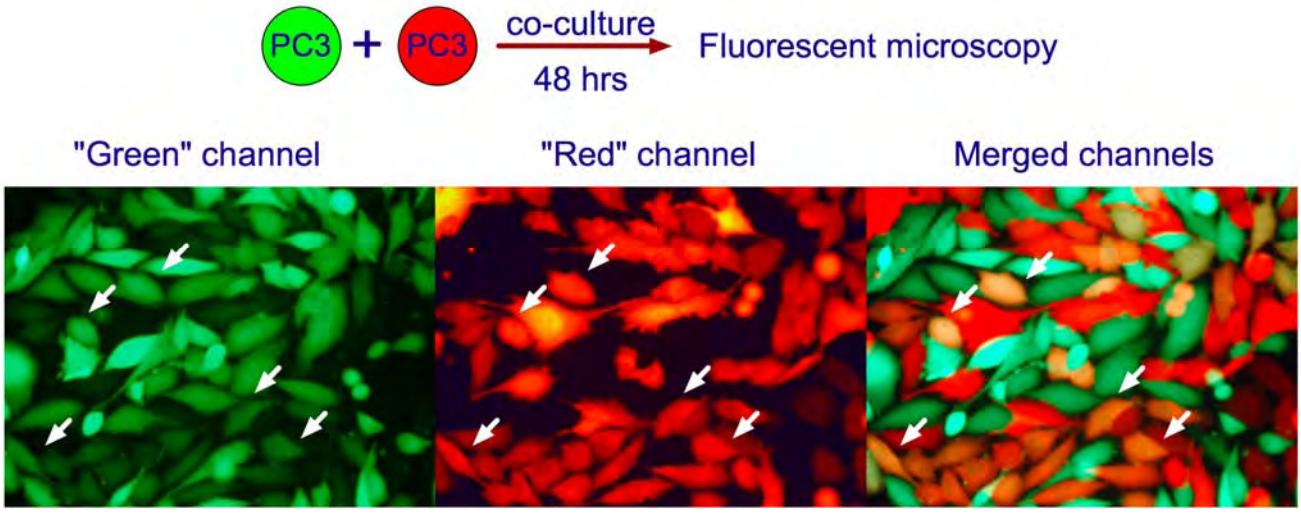

Figure 1. Prostate cancer PC3 cells exchange genetic markers in tissue culture. PC3 cells expressing EGFP ("green" cells) or RFP ("red" cells) were cultured together as indicated and analyzed by fluorescence microscopy. Some of the numerous cells that expressed both proteins are indicated with arrows.

possibility was that "…the mechanism of gene transfer is new, which will become apparent if we find that neither cell fusion nor apoptosis are involved. In this case we will investigate what this mechanism is using observations that we will accumulate by accomplishing this aim." During the first year of the funded research we have found that the third hypothesis is correct.

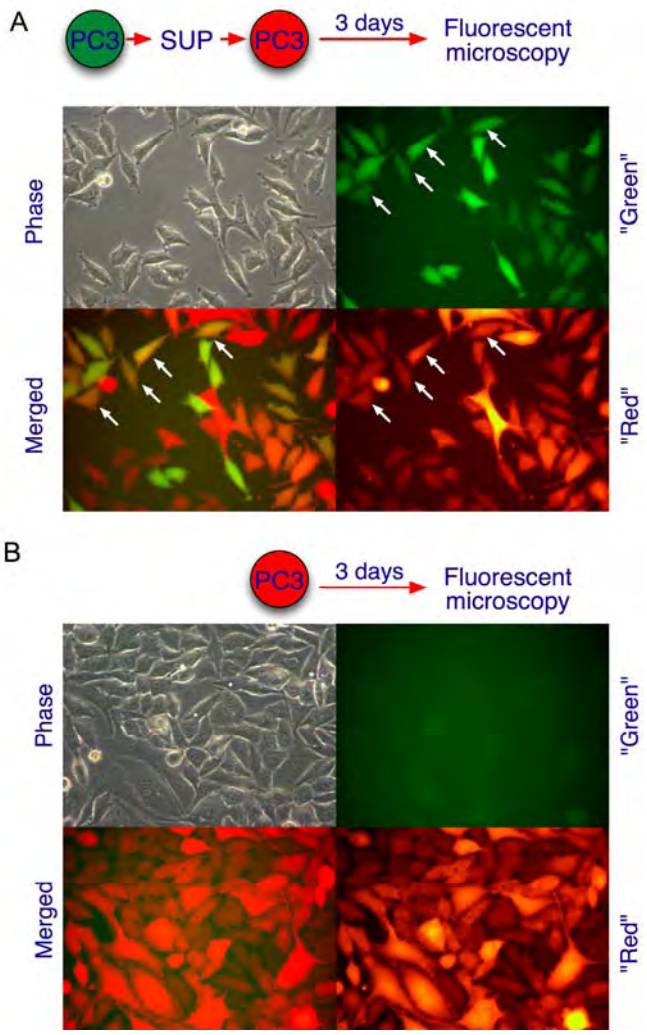

Figure 2. Genetic markers are transferred by a filterable activity. A. Tissue culture medium conditioned by "green" PC3 cells was passed through a .45 μ and added to "red" PC3 cells, which were analyzed by fluorescence microscopy in three days. "Red" PC3 cultured in normal medium were used as a control (B). Some of the cells in which expression of EGFP was induced are indicated by arrows.

Several observations led us to this conclusion. One, that co-culturing "red" and "green" PC3 cells produced cells that expressed both fluorescent proteins (Figure 1). However, we could not detect any instances of cell fusion by monitoring the cells by time-lapse microscopy or by detecting an expected increase in the number of binuclear or multinuclear cells, which are an immediate consequence of cell fusion. Therefore, we concluded that the mechanism of gene transfer was likely to be different from fusion.

The second observation came from testing whether the transfer of genetic material requires a contact between the cells and found that culturing "red" cells in a filtered tissue culture from "green" cells produced "yellow" cells (Figure 2). Therefore, we concluded that the cells secreted an activity that transferred genetic information and considered two explanations for what this activity might be.

The first explanation was that the transfer was mediated by engulfment of apoptotic bodies. This hypothesis appeared unlikely because we found the rate of apoptosis in PC3 cells to be too low to explain the incidence of gene transfer (Figure 1).

Prompted by our observations in an unrelated study, we hypothesized that *EGFP* and *RFP* were transferred by viruses. We reasoned that both genes were introduced into PC3 cells by retroviral vectors, which implied that a replication competent retrovirus that could for some reason infect PC3 cells could, in principle, transfer either *EGFP* or *RFP* by two mechanisms - by recombining with the vectors and thus acquiring the genes, or by packaging the RNA expressed by the vectors and thus transferring it into infected cells.

The viral transfer hypothesis predicted that the virus, which for the sake of convenience we called PC3V, from "green" or "red" PC3 cells should be able to propagate in other cells. If true, then tissue culture

medium of the infected naïve cells should contain the viruses that can infect another set of naïve cells. We tested this prediction (Figure 3) by incubating human fibroblasts with tissue culture medium conditioned by "green" PC3 cells, replacing the medium next day, and then allowing the fibroblast to condition the medium for three days. We then collected the conditioned medium, filtered through a .45µ filter, added to naïve fibroblasts and analyzed the cells in three days by fluorescent microscopy (Figure 3).

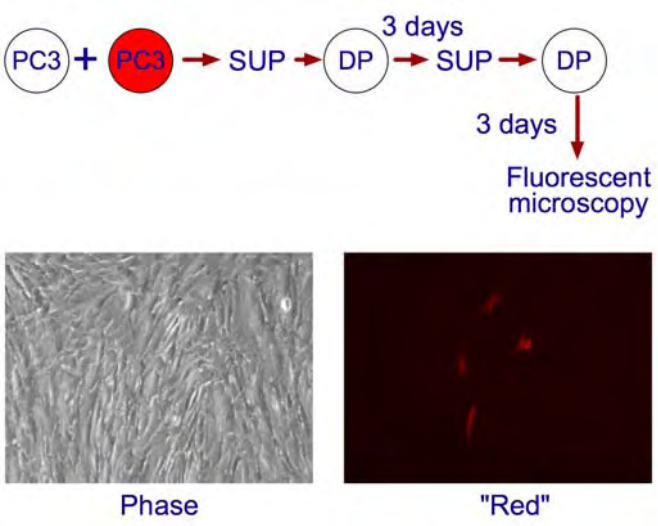

Figure 3. The activity that transfers genetic markers of PC3 cells has properties of a virus. Tissue culture conditioned by "red" PC3 cells was passed through a .45µ filter and added to human fibroblasts that expressed a dominant negative mutant of P53 (DP cells). The cells were washed with fresh unconditioned medium the next day and cultured for three days. The medium conditioned by the cells was applied to naïve DP cells, which were analyzed in three days, which revealed the expression of the RFP gene. This figure was provided by Dr. Glinsky.

The fibroblasts indeed expressed EGFP as was manifested by green fluorescence. Therefore, we concluded that the transfer of genetic markers between the cells was mediated by a virus, to which we will refer for the sake of convenience as PC3V. This experiment also demonstrated that PC3V can infect human cells other than PC3.

The results indicating that a virus is responsible for horizontal gene transfer were reproduced and extended by our collaborator Dr. Glinsky (Ordway Research Institute, Albany), who also documented gene transfer through tissue culture supernatant by flow cytometry and found that genes encoding EGFP and RFP could transfer independently of each other.

The model that a virus was surreptitiously transferring the genetic markers raised several questions. What was this virus? Where did it come from? Did it have oncogenic properties? Because knowing that identifying the virus would greatly help to find answers to the other questions, we began developing strategies to identify PC3V.

To decide how to proceed, we considered two scenarios. One, that PC3V was a retrovirus that recombined with the retroviral vector encoding EGFP or RFP. If true, PC3V could be identified by infecting naïve cells with the virus from "green" PC3 cells and then obtaining the sequence of the DNA adjacent to the integrated virus, which could be done by using *EFGP* as a starting template. Another scenario was that P3V packaged the RNA expressed by the vector without recombining with it. In this case sequencing the genome adjacent to EGFP could be uninformative, as it would not reveal PC3V. However, purifying the virus secreted by the cells and identifying it by peptide sequencing could be more successful. Given our experience in the latter approach, we decided to purify and identify PC3V.

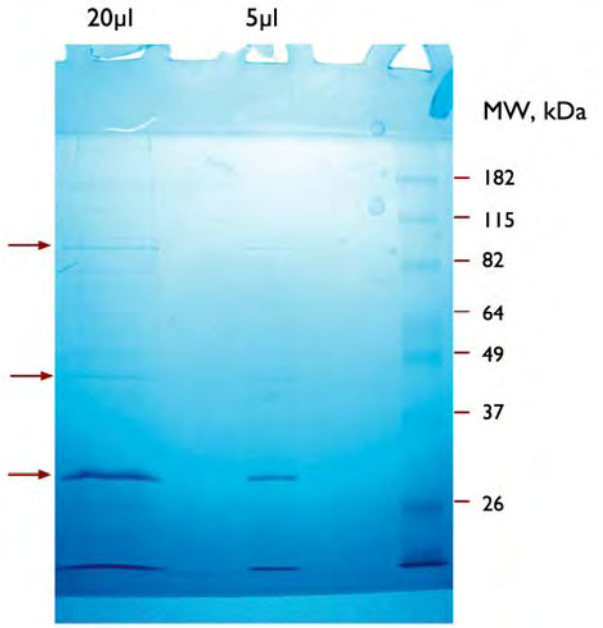

Figure 4. Polypeptides purified from medium conditioned by "green" PC3 cells. "Green" PC3 cells were cultured in the medium containing equal volumes of DMEM and F12 and no serum for three days. The medium was collected, passed through a .45µ filter, clarified by centrifugation at 1000g, the remaining particulate material was pelleted at 100,000g, resuspended in SDS sample buffer, fractionated by electrophoresis (5 µl or 20 µl of the 100 µl sample were loaded) and stained with Coomassie. The polypeptides indicated by the arrows were sent out for sequencing.

To facilitate the purification, we chose a serum-free and protein-free medium in which viable PC3 could live for several days and still secrete the virus. Using this medium, we obtained a preparation of the infectious activity and analyzed it by gel electrophoresis (Figure 4). The analysis revealed three major polypeptides that were identified by preliminary peptide sequencing as fragments of Gag and Env of the mouse leukemia virus (MuLV). If confirmed, these results would raise two possibilities.

One possibility is that PC3 cells injected into mice were infected with MuLV. MuLV exist as complex populations that include xenotropic viruses, which infect species other than the mouse, including humans. We found that PC3V does not infect mouse 3T3 cells, consistent with a possibility that PC3V is related to xenotropic MLV. If this conclusion is true, then our results would suggest that a mouse virus could transfer genetic information in animal models between the xenograft and the host and perhaps, between the xenograft and the researchers who do the experiments with genes that far less innocuous than *EGFP*. Our results would also emphasize the need to consider horizontal gene transfer in interpreting experimental results, especially if retroviral vectors are used.

The second possibility is that PC3V is only closely related to MuLV, but is not MuLV itself. This hypothesis was suggested by the surprising finding (Dong et al., 2007; Urisman et al., 2006) that some human prostate cancers contain a virus which is closely (about 95% nucleotide identity) related to xenotropic MuLV and, accordingly, was named xenotropic MuLV-related virus (XMRV). The origin or the consequences of XMRV infection are unclear, which suggests that PC3V had been latent in PC3 cells and was induced after the cells were injected into the mouse. Given the close sequence homology between MuLV and XMRV, determining a relationship between PC3V and MuLV will require sequencing PC3, an effort that is now under way. Since the function and the origin of XMRV is practically unknown, our results might help to understand better a possible link between viruses and prostate cancer.

Overall, we accomplished Aim 1 by identifying the mechanism of horizontal gene transfer between prostate cancer cells. We think that continuing this unexpected line of research until the virus is identified will be informative by providing new insights into how viruses might be related to prostate cancer and by characterizing a new way of horizontal gene transfer in animal models of cancer.

AIM 2. To determine whether cell fusion affects metastatic properties of prostate cancer cells. The main goal of this aim is to test whether fusion of prostate cancer cells to themsleves or to normal cells of the host affects the rate or the tropism of metastasis. By design, research proposed in this Aim is independent from the results obtained in Aim 1. However, the unexpected finding that viruses, which we planned to use to fuse cells, could be involved in horizontal gene transfer, led us look for another

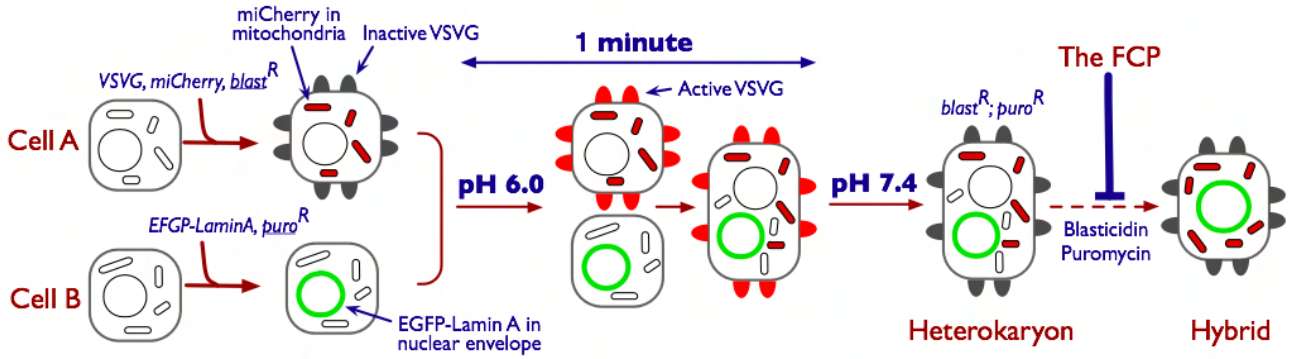

Figure 5. The fusion assay. Fusion partners (cells A and B) are transduced with one of two retroviral vectors. One will confer resistance to blasticidin (*blast*^{R}) and carry the gene encoding VSVG, the fusion protein of the vesicular stomatitis virus, and the gene encoding a mitochondria-targeted fluorescent protein Cherry (miCherry). The second confers resistance to puromycin (*puro*^{R}) and carry a gene encoding a fusion between the fluorescent protein EGFP and Lamin A, which localizes to nuclear lamina. The cells are plated together and the medium replaced with PBS at pH 6, which ***reversibly*** activates the fusogenic activity of VSVG, thus initiating fusion of adjacent cells. After one minute of incubation the cells are washed with normal culture medium, which makes VSVG inactive. The heterokaryons can be identified by fluorescence microscopy, as shown, or by phase contrast microscopy.

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approach to cell fusion.

We ruled out the commonly used polyethylene glycol (PEG) because of its toxicity and the difficulty to control for its effects unrelated to cell fusion. We also decided against using inactivated Sendai virus because we had no facilities to produce this virus and were concerned that this virus is pathogenic or even fatal in mice, which could be a problem if we fail to inactivate this virus completely. Therefore, we developed an approach (Figure 5) in which cells are fused with ectopically expressed VSV-G, the fusogenic protein of the vesicular stomatitis virus. VSV-G is practically inactive at physiological pH, but is

rapidly and reversibly activated at pH 6 or below, fusing cells of a variety of species within one minute. We found that cells transduced with *VSV-G* express sufficient amounts of the protein to cause cell fusion at pH 6, but otherwise appear unaffected if cultured at normal pH. The heterokaryons and hybrids could be identified by fluorescent or drug selection markers (Figure 5). An additional benefit of this approach is that VSVG fuses cells of various types and species, including human and mouse and is amenable to developing an inducible system to fuse cells *in vivo*. Therefore, to accomplish Aim 2 we will use this approach.

Another change that we plan was suggested by our experience acquired after this application was funded. We found (Duelli and Lazebnik, 2007) that fusion of premalignant cells could produce highly aggressive tumors that disseminated even if injected subcutaneously. Therefore, in the initial experiments we plan to use subcutaneous injection instead of injecting the cells into the prostate. As a result, we will simplify the experiments technically by avoiding the need for surgery, which requires the expertise of our collaborator Dr. Glinsky (Ordway Research Institute, Albany). By avoiding surgery, we will also simplify the logistics of the experiments as we will be able to conduct them in their entirety at CSHL.

Dr. Glinsky will remain on this project as our collaborator, who agreed to consult us by contributing his expertise in prostate cancer in general and in animal models in particular.

REPORTABLE OUTCOMES: In the attached invited review we developed further our ideas about a potential link between viruses and cancer. We also generated a series of plasmids and cell lines that will be made available to the scientific community once our results are reported. We already distributed some of the cell lines to fulfill requests that followed public presentation of our results.

CONCLUSIONS: Overall, by accomplishing Aim 1 we unexpectedly entered an area of cancer biology – a relationship between viral infections and cancer – from a perspective that may be unrelated to cell fusion but might provide new insights into an intriguing link between human prostate cancer and viruses closely related to mouse leukemia virus. We plan to continue research in the framework of this Aim until the virus is unambiguously identified and then seek further funding which origin will depend on the results. Our findings also led us to reconsider the technology that we planned to use to produce cell hybrids and to develop new technology, which will be central to accomplishing Aim 2, the central focus during the remaining years of the research funded by this grant.

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APPENDICES:

Duelli & Lazebnik, Nature Cancer Biology will be attached to the pdf file

OPINION

Cell-to-cell fusion as a link between viruses and cancer

Dominik Duelli and Yuri Lazebnik

Abstract | The ability to fuse cells is shared by many viruses, including common human pathogens and several endogenous viruses. Here we will discuss how cell fusion can link viruses to cancer, what types of cancers it can affect, how the existence of this link can be tested and how the hypotheses that we propose might affect the search for human oncogenic viruses. In particular, we will focus on the ability of cell fusion that is caused by viruses to induce chromosomal instability, a common affliction of cancer cells that has been thought to underlie the malignant properties of cancerous tumours.

Over the past 50 years cancer research and clinical oncology have produced ~1,600,000 publications, but despite numerous successes this colossal effort has had little effect on overall cancer mortality[1,2]. Looking to the history of medicine for an explanation one can find that a failure to cure an apparently complex disease was sometimes caused by overlooking its primary, and often simpler than expected, cause. A recent example is peptic ulcer, which was a common, debilitating and sometimes fatal disease that for decades was believed to be a complex multifactorial psychosomatic disorder caused by urban and familial stress[3]. This model led to drugs that managed the disease, but the discovery that the primary cause is a bacterial infection revealed that the model was a convincing myth and that most ulcers could be routinely cured and prevented by a course of available antibiotics[4].

The current view of cancer is that of a complex disease caused by accumulating genomic and epigenetic aberrations that affect a defined set of cellular properties[5]. This view focuses research on treating cancer rather than on preventing it. Indeed, avoiding or even identifying causative mutagens that range from cosmic radiation to spontaneous chromosomal aberrations is not always practical or even possible.

Yet a primary cause of several cancers, which together account for about one-fifth of all cancer cases in the world, is a defined virus or bacterium[6]. Remarkably, vaccinating against some of these pathogens effectively prevents the malignancies they cause[7,8], thus adding cancer to the list of incurable diseases, such as smallpox, that are preventable by immunization. The success of vaccination draws attention to the view that more cancers than we think might be caused by infectious agents and thus could be prevented by identifying and neutralizing these pathogens[6]. Identifying new oncogenic pathogens seems an even more attractive strategy after considering that human cancers caused by viruses have no overt hallmarks of their viral origin and that for some of these cancers the evidence for non-viral causes had been sufficiently compelling to dismiss a viral origin as highly improbable[9,10].

However, if known causal relationships between viruses and human cancer are any indication, new relationships will be difficult to reveal even if they exist[6,11]. Indeed, human viruses cause cancers in only a minority of the infected people and do so after decades of latency, which can frustrate the epidemiological approach, a primary tool for identifying infectious pathogens. This tool is blunted further if only some strains of a virus are oncogenic or if the virus is ubiquitous. A virus might also be overlooked if it does not harm the infected cells, makes only some of them cancerous, works in cooperation with other carcinogens or is not produced at all by the cancers it caused. One approach to bypass these difficulties while explaining their origin is to identify viral activities that contribute to cancer development and then scrutinize human viruses that have them. We[12] and others[13] proposed that one of these activities is the ability of viruses to fuse cells.

Viruses and cell fusion

Enveloped viruses, which include common human pathogens and most of the known oncogenic viruses, enter cells with the help of viral proteins that fuse biological membranes[14,15]. A well-known consequence of this mechanism is the ability of viruses to fuse cells (FIG. 1), both *in vitro* and *in vivo*, thus producing heterokaryons, cell hybrids and syncytia[16]. Some non-enveloped viruses also express proteins that fuse cells, which is thought to facilitate viral spread[17]. Overall, viruses that can fuse cells (fusogenic viruses) are nearly ubiquitous in humans (see Supplementary information S1 (table)), suggesting that accidental fusion in the body is not uncommon. However, the incidence has not been investigated because this event is presumed to be largely harmless and has even been explored as a tool for cancer therapy[18].

However, viruses fuse cells indiscriminately, in contrast to the physiological fusion of differentiated cells. The latter is tightly controlled and restricted to only a few cell types which, with the exception of fusion of gametes or stem cells, produce only terminally differentiated, non-proliferating heterokaryons[19,20]. Therefore, most cells made by accidental fusion are likely to be abnormal. This conclusion is supported by what is known about hybrids made by treating cells with inactivated viruses or fusogenic chemicals *in vitro*, which essentially recapitulates accidental fusion occurring in the body[21]. The abnormalities of these hybrids include an unstable genome, unstable gene expression and properties not found together in a normal cell[22,23]. The observation that these features are shared with cancer cells led to the proposal that accidental cell fusion can contribute to cancer development in two ways: by destabilizing the genome and by changing gene expression, sometimes in an apparently random way.

The natural ability of cell fusion to make tetraploid cells and a well documented but unexplained propensity to trigger chromosomal instability suggested the idea[24] that accidental cell fusion causes cancer through mechanisms described by the tetraploidy model of carcinogenesis[25–27]. This model argues that cells can become cancerous by first becoming tetraploid and then undergoing a period of chromosomal instability (CIN), a poorly understood condition manifested by the propensity of cells to incur numerical and structural chromosomal aberrations, resulting in aneuploidy.

A long-standing view[28–30] is that, by rare chance, chromosomal instability produces cells with properties that are sufficient to avoid various tumour-suppressor mechanisms and with genomes that are stable enough to produce progeny sufficient to form a tumour. In essence, this view equates carcinogenesis with an effective approach of modern drug discovery — making a library of randomly synthesized chemical

compounds and then screening it for a drug with required properties — except that the chance of making a cancerous cell might be even lower than that of making a certain drug. Tetraploid cells are thought to have a higher chance of becoming cancerous than diploid cells because they might be more prone to chromosomal instability, might generate larger and more diverse 'libraries' of abnormal chromosome complements and could be less likely to die by losing all copies of a chromosome[31]. How cells become tetraploid during cancer development, how tetraploidy causes chromosomal instability and how this instability contributes to cancer development is still uncertain, but the ploidy of many solid cancers and the presence of tetraploid cells in premalignant lesions[25] is consistent with the model, as is chromosomal instability in some cancers[32–34].

CIN generates diverse populations of abnormal cells not only by causing chromosomal aberrations, but also through cascading genome-wide changes in gene expression triggered by these aberrations[35,36]. The ongoing cell fusion in the populations can increase the diversity by combining and recombining the genomes, a mechanism homologous to sexual reproduction, a powerful engine for creating and sustaining diverse populations that relies on cell fusion[13].

The fundamental ability of cell fusion to combine properties of distinct cells (a required part of sexual reproduction) suggested a hypothesis[37–39], which is yet to be tested definitively, that tumour cells can acquire the capacity to metastasize by fusing to the cells of the target tissues, such as bone-marrow cells[40]. Acquiring new properties by fusing to the cells that have them was suggested as a mechanism by which tumour cells acquire properties of stromal cells, perhaps facilitating tumour invasion[41]. Such a 'marriage of convenience' is reminiscent of an extensively documented phenomenon: that bone-marrow stem cells, for unknown reasons, can fuse *in vivo* to differentiated cells, producing proliferating hybrids that have properties of these differentiated cells[23,42–44]. In one study, bone-marrow cells injected into mice could even form gastric cancer[45]. The authors found no evidence of fusion between the injected and host cells but fusion as a mechanism has remained a suspect[46]. That the similarity between the observed stem-cell fusion and the marriage-of-convenience hypothesis might be more than superficial was suggested by a recent observation that osteoclasts of myeloma patients contain nuclei of myeloma cells[47], which might explain why metastases often dissolve bone, and by reports that primary cancer cells or tumour cell lines grafted into rodents fuse to the host cells[41,48–50].

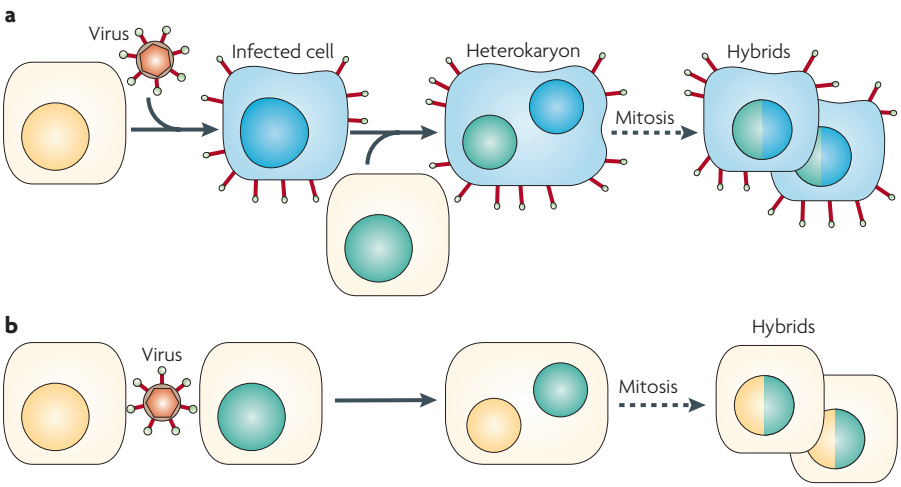

Figure 1 | **Viruses fuse cells by two mechanisms. a** | Fusion from within occurs after a virus infects a cell and expresses its fusogenic protein(s) (red), which are localized at various cellular compartments, including the plasma membrane. As a result, the cell can fuse to any other cell that has a receptor for the fusion proteins. The cell containing nuclei of the fusion partners (a heterokaryon) can enter mitosis, which produces mononucleated cells (hybrids), or remain quiescent. Fusion from within also occurs if only a viral fusogenic protein rather than the whole virus is expressed. For example, Env proteins of endogenous retroviruses are sufficient to fuse cells and are expressed in some normal and cancerous tissues. **b** | Fusion from without is mediated by viral particles that fuse cells without infecting them. How this fusion occurs is not entirely clear and is thought to involve bridging of the cells by the particles. A significant difference between the two mechanisms is that fusion from within produces infected cells, while fusion from without can produce hybrids that carry no traces of the virus that made them. As a result, viruses can produce hybrids that have no indication of their viral origin.

Although cell fusion can combine some properties of parental cells, the resulting hybrids, like children, who are also a progeny of cell fusion, are more than an average of their parents. Indeed, cell fusion triggers massive changes in gene expression, referred to as reprogramming, even in hybrids with a stable genome or in quiescent heterokaryons[43,51–53]. The phenomenon of reprogramming and its implications are still poorly understood for accidental fusion, and even for stem-cell fusion, which is being actively pursued for stem-cell-based therapies[54]. Therefore, although cell fusion is similar to other mechanisms of lateral DNA transfer that might mediate exchange of genetic information between tumour cells[55,56], the transfer caused by cell fusion involves the entire genomes and can induce genome-wide epigenetic changes whose mechanisms and consequences are largely unknown.

Considering the potential relationships between cell fusion and cancer and the fact that viruses fuse cells in the body, we[12,57] and others[13] proposed that viruses contribute to cancer development by fusing cells. Several observations suggested how this causal link might work.

Observations

We found that fusion of normal differentiated human cells by an infectious virus, but not the infection itself, caused cell-cycle arrest[12]. This observation led us to propose that a cell-cycle checkpoint, which we call for convenience the fusion checkpoint (FCP), prevents proliferation following accidental fusion, thus making fusion harmless. What this checkpoint is, how it is regulated and how it is related to known checkpoints is unclear, in part because the mechanisms ensuring that cells that are formed by physiological cell fusion do not proliferate are poorly understood. We also found, however, that if even one of the two fusing cells expressed the adenoviral oncogene E1A, or a mutated form of the tumour suppressor p53, then the resulting hybrids proliferated. Therefore, we concluded that oncogenic events can enable proliferation of accidentally fused cells, perhaps by deregulating the FCP.

We noticed that while the parental cells had relatively uniform karyotypes, the karyotypes of hybrids were diverse and changed over time, indicating that cell fusion caused chromosomal instability[57]. The number of chromosomes in the hybrids ranged from 34 to 184. Consistent with the expected consequences of CIN, the hybrids were

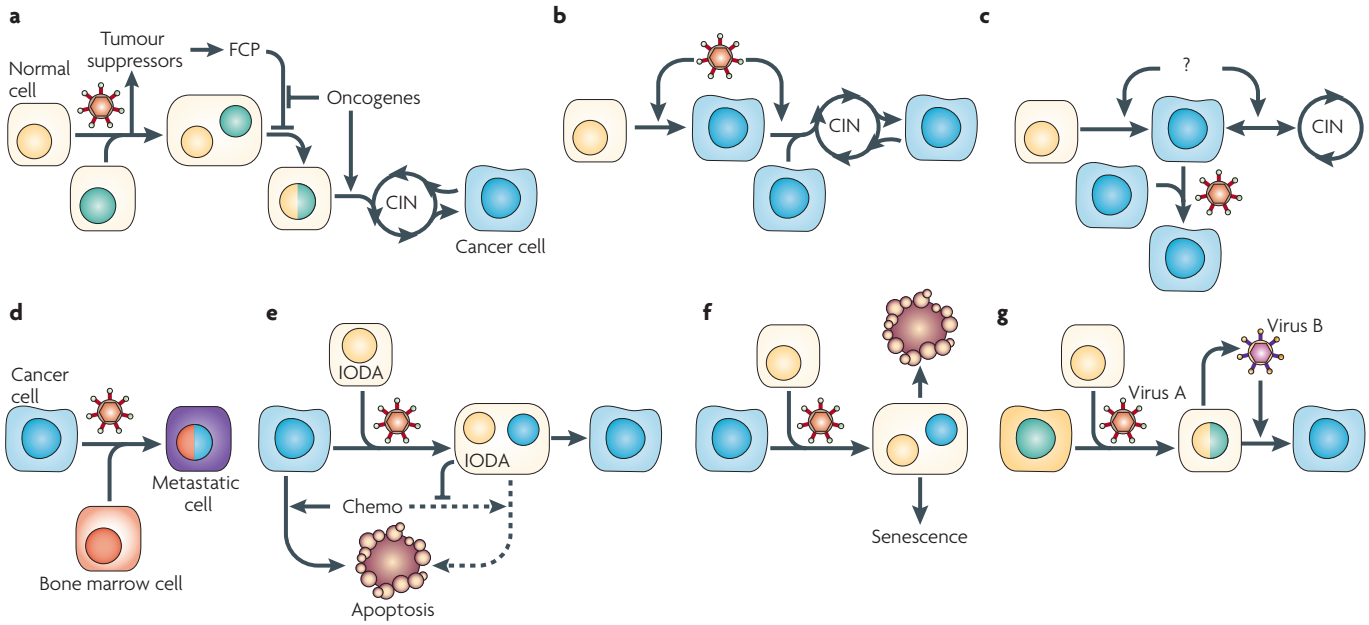

Figure 2 | **How cell fusion caused by viruses might relate to cancer initiation or progression — several conjectures. a** | The synergy hypothesis suggests that fusion of normal differentiated cells causes cell-cycle arrest, mediated by the fusion checkpoint (FCP), which is regulated by tumour suppressors and prevents the heterokaryons from entering mitosis. However, if the cell cycle of even one of the parental cells is deregulated, for example, by the expression of an oncogene, then the heterokaryon enters mitosis, which yields mononuclear proliferating cells that are affected by chromosomal instability (CIN), a condition that can produce cancerous cells. **b** | The byproduct hypothesis suggests that a virus that causes cancer by a mechanism that is unrelated to cell fusion also fuses cells by accident, thus causing CIN, which contributes to cancer progression. **c** | The coincidence hypothesis proposes that cancer cells derived by various mechanisms are conducive to viral replication, including that of viruses that fuse cells with or without any effect on tumour progression. **d** | The marriage-of-convenience hypothesis suggests that by fusing tumour and normal cells viruses can provide cancerous cells with properties that enable travel throughout the body and proliferation at distant sites. **e** | The escape hypothesis proposes that fusion to normal cells might also render cancerous cells temporarily insensitive to chemotherapy (chemo). This proposal stems from the observations that normal cells have an inhibitor of oncogene-dependent apoptosis (IODA) which inhibits the mitochondrial permeabilization that is required for apoptosis. **f** | The suppression hypothesis suggests that fusing to normal cells might inactivate the tumorigenic potential of a cancerous cell, thereby making this cell harmless. **g** | The reactivation hypothesis is suggested by the reports that cell fusion can reactivate silent viruses and proposes that some of these viruses can be oncogenic.

diverse in respect to all the properties that we evaluated, such as morphology, cell-cycle duration, the rate of spontaneous apoptosis and the expression of the genes that we analysed. These observations led us to conclude that viruses can cause chromosomal instability by fusing cells whose cell cycle is deregulated and that this instability and perhaps other consequences of fusion are sufficient to produce libraries of abnormal and proliferating cells.

Considering the model that chromosomal aberrations can cause cancer, we tested whether any of the hybrids that we made were carcinogenic. Indeed, we found that some of the hybrids, but not the parental cells, produced aggressive and evolving cancers in nude mice[57]. The karyotypes of these tumours were indistinguishable in their chromosome number, complexity, ability to evolve and the types of aberrations from those of some solid human cancers. The karyotypes of the tumours were distinct from the karyotypes of the injected cells and were more uniform, indicating that tumour progression was associated with selection of some cells. Therefore, we concluded that by fusing cells viruses can produce populations of abnormal cells that are affected by chromosomal instability and are sufficiently diverse to include cancerous cells. Our observations suggest several conjectures.

Conjectures

The synergy hypothesis. This hypothesis states that the events that we observed in the laboratory recapitulate the development of some human cancers (FIG. 2a). This view implies that these cancers result from a synergy of two events: cell-cycle deregulation and cell fusion. The first event inactivates cell-cycle checkpoints, including that activated by cell fusion (the putative FCP), and might result in cells with relatively stable genomes that are characteristic of benign neoplasia. Infectious exogenous or induced endogenous viruses then fuse these cells, thus triggering chromosomal instability and the consequent emergence of cancerous cells. In essence, the recapitulation hypothesis states that by mating cells that have an abnormal cell cycle, viruses create a literal breeding ground for cancerous cells.

For example, human papillomavirus (HPV), the causative agent of most cervical cancers, expresses oncogenes E7 and E6 in the epithelial cells of the cervix which deregulate the cell cycle and prevent apoptosis, but are insufficient to make cells cancerous. The synergy hypothesis suggests that a virus, such as herpesvirus, which has been considered a cofactor in cervical carcinogenesis[6], would fuse cells infected with HPV, producing tetraploid or polyploid cells observed in cervical premalignant lesions[58]. Cell fusion then causes chromosomal instability with the consequent emergence of aneuploid cancerous clones that may have an unstable or relatively stable chromosome complement in the triploid to tetraploid range[59]. The chance that these clones emerge will depend on how frequent cell fusion is and how often this fusion can produce a cancerous cell. A similar scenario can be envisioned for other cancers with increased ploidy and complex karyotypes,

Table 1 | **Human oncogenic viruses**

Family	Human oncogenic virus	Cancer association	Causes cell fusion?	Cytopathological evidence of tetraploidization?	Has proteins that deregulate cell cycle?
Flaviviridae	Hepatitis C virus	Hepatocellular carcinoma (increased incidence of lymphoma)	Yes	Yes[130]	Yes
Hepadnaviridae	Hepatitis B virus	Hepatocellular carcinoma	Yes	Yes[130]	Yes
Herpesviridae	Epstein–Barr virus (HHV-4)	Burkitt lymphoma Hodgkin lymphoma	Yes	Near diploid[131,132]	Yes
		Naso pharyngeal carcinoma Gastric carcinoma	Yes	Yes[131,132]	
	Kaposi sarcoma virus (HHV-8)	Kaposi sarcoma	Yes	Primarily near diploid	Yes
Retroviridae	Human T-lymphotropic virus 1	Adult T-cell leukaemia or lymphoma	Yes	Primarily near diploid	Yes
Papillomaviridae	Human papillomavirus	Anogenital cancers	Unknown	Yes[58]	Yes

which includes cancers of the oesophagus[34], breast[60], colon[32,61] and pancreas[62]. This speculative mechanism is, of course, only one of several that can explain the aneuploidy of cervical and other cancers[63,64].

Cell-cycle deregulation might be unnecessary if viruses fuse stem cells[44,65], for which cell-cycle regulation is more plastic than that of somatic cells[66]. Despite the therapeutic interest in stem-cell fusion[54,67], whether such hybrids are genomically stable is still unclear. Some reports have shown that aneuploidy results[68], which is consistent with chromosomal instability as a hallmark of cell hybrids.

The synergy hypothesis predicts that by causing cell fusion viruses can contribute to cancer in various ways. They can cooperate, as suggested for HPV and herpesvirus, or a single virus can both fuse cells and deregulate their cell cycle either by carrying an oncogene or by insertional mutagenesis. The synergy hypothesis implies that more than one fusogenic virus can trigger chromosomal instability, thus giving a new perspective to consider in the ongoing search for oncogenic viruses, which is largely based on the assumption that a single virus causes cancer in a particular tissue[69,70]. For example, the ongoing debate about a viral origin for human breast cancer focuses on how often a particular virus is found in these tumours[71,72]. The synergy hypothesis suggests that breast cancer and premalignant lesions could be tested to see if they are associated with fusogenic viral activity rather than with a particular virus.

How often cancers contain fusogenic viruses and what these viruses are has not been investigated systematically, but the few published studies suggest that fusogenic activity in tumours is not uncommon. One study found that primary cells from each of 30 ovarian cancer patients formed syncytia, whereas cells from patients with benign tumours did not[73]. Human cancer cells explanted into rodents were reported to form hybrids with the host cells[41,48–50] and secrete virus-like particles[73]. We found that cells from 7 out of 28 cancer cell lines that we tested can fuse to other cells and secrete a particulate fusogenic activity[57].

That cells in cancers can fuse was also indicated by detecting premature chromosome condensation (PCC), which is the condensation of interphase chromosomes following fusion between an interphase and a mitotic cell[74]. PCC could also be caused by events other than cell fusion, but cells in which PCC is caused by cell fusion are distinguished as those having proper mitotic and prematurely condensed chromosomes in a single cell. Because mitotic cells usually constitute less than a few percent of a proliferating population, PCC is rare even in experimental systems of cell fusion. Nevertheless, PCC was detected in haematological cancers[75–77], as well as in breast[75,78], colon[79], bladder[75,80], cervical[81], gastric[78], ovarian[78] and pancreatic cancer[82], and was suggested to indicate the presence of fusogenic viruses in tumours[83]. Neither the cause nor consequences of the fusion manifested by PCC in these cancers is known.

Cell fusion, whether due to viruses or other causes, is also suggested by the presence of pleomorphic giant cells (PGCs), which are multinuclear cells derived from tumour cells[84,85] and found in some malignancies, most frequently in pancreatic cancers. Whether PGCs result from cell fusion or by failing to divide is unclear, but having numerous nuclei suggests that these cells are syncytia.

The synergy hypothesis implies that viruses do not need to be infectious to contribute to cancer development and that even expression of a single viral or cellular fusogenic protein, such as the retroviral Env, may be sufficient. These implications might provide a new explanation for the intriguing and puzzling observations that at least the Env protein of human endogenous retroviruses (HERV), which are non-infectious, are often expressed in human cancers[86]. Because these proteins fuse cells, they can increase their ploidy and trigger chromosomal instability. HERV-K, or its Env[87], is expressed in most adult germ-cell cancers[88,89], nearly all of which are triploid to tetraploid[90], as would be expected of hybrids. HERV-K Env is also expressed in many melanomas[91,92], which often have complex karyotypes of similar ploidy[93,94], as well as in breast[95], ovarian[96] and prostate[97] cancers. HERV-W Env, also known as ERVWE1 and syncytin 1 (REFS 98,99), is normally expressed only in the placenta, where it is thought to fuse trophoblasts during pregnancy. However, it has also been found in breast[100] and endometrial cancers[101] whose ploidy falls into two groups, nearly diploid and triploid to tetraploid[60,102]. It would be interesting to test whether syncytin 1 is predominantly expressed in the non-diploid cancers, as the synergy hypothesis predicts.

In principle, accidental cell fusion does not need to involve viruses to contribute to cancer, but rather may result from deregulation of fusogens that mediate physiological cell fusion. Evaluating this possibility will require learning more about physiological cell fusion, which is still surprisingly poorly understood, and identifying fusogenic proteins that mediate it

in mammals, as syncytin 1 is the only such protein currently known.

One argument that is often raised against the synergy hypothesis is that cancers are monoclonal, meaning that they develop from one cell, whereas cell fusion involves at least two. This argument is easier to analyse by considering the evidence underlying the notion that human cancers are monoclonal, which is extensively reviewed elsewhere[103,104]. This evidence is based primarily on the fact that cells from females have one of their two X chromosomes randomly inactivated[104]. Which chromosome is active can be determined by analysing the ratio of expressed alleles of a gene on the X chromosome. Because the inactivation is random, normal tissues are expected to contain an equimolar ratio of the alleles. However, if a tumour is a progeny of a single cell then one of the alleles should predominate in the tumour tissue, which indeed has been found in many, but not all, tumours[104].

One allele, however, would also predominate in hybrids derived from the fusion of two cells with identical active alleles. This is a likely event, considering that fused cells have to be next to each other, especially if the fusion occurs in a premalignant lesion that is already monoclonal or is made of patches of monoclonal cells. A single allele could also predominate if one of the active X chromosomes is lost during the chromosomal instability that follows cell fusion. Recent reports on unexpected changes in X-chromosome activity in breast and ovarian cancers, including the possibility of reactivation[105], and on discordance between primary tumours and metastases in melanoma[106] might suggest other explanations that could reconcile the behaviour of X chromosomes in cancer cells and the synergy hypothesis, especially considering the lack of systematic studies on X-chromosome activity in somatic hybrids.

Another argument against the synergy hypothesis is the lack of epidemiological evidence that fusogenic viruses are associated with a particular cancer or cancers. One can argue, however, that epidemiological evidence, even for human cancers that are induced by viruses (with the exception of human T-lymphotropic virus type 1 (HTLV1)), became apparent only after other evidence indicated a viral cause. If viruses indeed cause cancer by fusing cells, epidemiological evidence would be even more difficult to analyse as the ability to fuse cells is shared by numerous and common human pathogenic and commensal viruses,

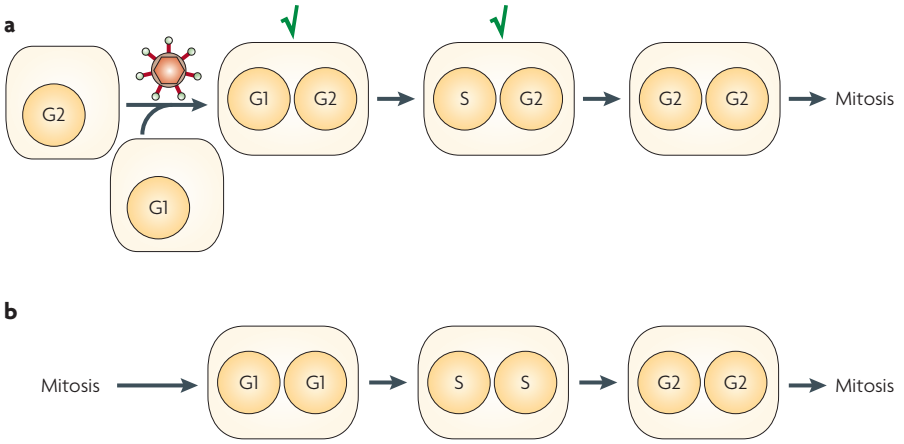

Figure 3 | **How can one distinguish binuclear cells made by fusion rather than by failed cytokinesis?** If a binuclear or multinuclear cell is made by fusion (**a**), its nuclei can be asynchronous in their cell-cycle position, at least for some time. For example, fusion of two cells of which one is in the G1 phase of the cell cycle and the other is in the G2 phase would result in a heterokaryon with different amounts of DNA in its nuclei until the G1 nucleus also completed replication of its DNA and reached G2. However, if a binuclear cell is a result of failed cytokinesis (**b**) its nuclei will always be synchronous. Therefore, finding that the nuclei of a binuclear cell differ in their DNA content or in another phase-specific marker of the cell cycle would indicate that this cell is likely to be a product of cell fusion (green check).

and because the consequences of cell fusion caused by viruses has not been systematically investigated. Furthermore, with the exception of HPV, whose fusogenicity is yet to be tested, all human oncogenic viruses (Epstein–Barr virus, HTLV-1, hepatitis C virus and hepatitis B virus) are fusogenic (TABLE 1), but the role of this activity in carcinogenesis is unknown.

Overall, the synergy hypothesis suggests that identifying viruses that cause cell fusion in premalignant lesions and cancers should uncover the primary causes of some malignancies, and might also indicate how these viruses cause cancer.

The byproduct hypothesis. This hypothesis proposes that some viruses make cells cancerous by mechanisms that are unrelated to cell fusion, but also accidentally fuse cells, thus triggering chromosomal instability and revealing a viral aetiology for this process (FIG. 2b). In this case, identifying viruses that cause cell fusion in neoplasia would also reveal a primary cause of both malignancy and chromosomal instability, though not necessarily identify how the viruses cause cancer.

The coincidence hypothesis. The coincidence hypothesis suggests that fusogenic viruses that are unrelated to cancer tend to propagate in cancer cells, perhaps because a deregulated cell cycle makes these cells permissive for viral replication. Any accidental fusion contributes to chromosomal instability, but this is primarily caused by other processes (FIG. 2c). The coincidence hypothesis implies that finding viruses that accidentally fuse cells in tissue would be of value if the contribution of these viruses to chromosomal instability, or other properties of cells, affects cancer progression.

The marriage-of-convenience hypothesis. This concept postulates that irrespective of the causes of cancer, fusogenic viruses can make cancer cells metastatic by fusing them to cells of the target tissue, such as bone-marrow cells (FIG. 2d). Combining the skills of the fusion partners might produce tumour cells that can reside and proliferate in bone or other sites of metastasis. This hypothesis provides one explanation for metastasis and is consistent with some evidence from early studies[39,40,107]. In addition, it is consistent with the finding that osteoclasts from patients with multiple myeloma contain nuclei from myeloma cells[47]. However, this hypothesis is yet to be tested definitively using the tools of modern biology.

The escape hypothesis. This hypothesis states that tumour cells can temporarily escape chemotherapy by fusing to normal cells (FIG. 2e). This is based on our observation that human cells that die by apoptosis as a result of chemotherapy became temporarily resistant to the treatment after fusing to normal human cells[108]. We have suggested that

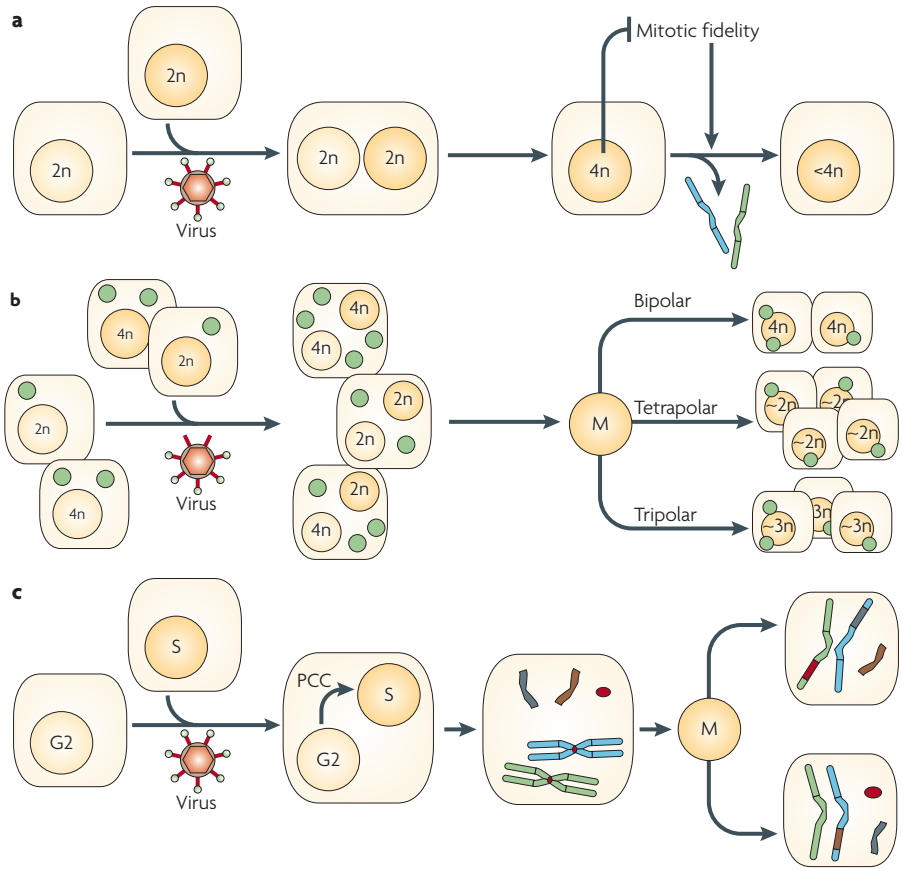

Figure 4 | **How does cell fusion cause chromosomal instability? a** | The overload hypothesis states that tetraploidy caused by cell fusion overwhelms the mitotic machinery, thereby decreasing its fidelity. **b** | The centrosome hypothesis suggests that the abnormal number of centrosomes (green dots) resulting from combining two or more cells having one or two centrosomes causes bipolar, tripolar, tetrapolar or asymmetric mitoses that divide chromosomes with low fidelity. In particular, a tripolar mitosis is likely to produce aneuploid cells in the triploid range, as eight sets of chromosomes have to be divided among three cells. As a tetrapolar mitosis can produce near diploid cells, cell fusion might produce not only the commonly expected tetraploid cells but also cells in which the chromosome complement is nearly normal. **c** | The conflict hypothesis proposes that chromosomal instability is caused by premature chromosome condensation (PCC), which is a consequence of fusion between an interphase and a mitotic or pre-mitotic cell. The chromatin condensation activity of the mitotic cell also condenses chromatin of the interphase nucleus, which might cause DNA breaks, especially if the interphase nucleus undergoes DNA replication, a process that is associated with multiple DNA breaks. The DNA fragments produced by PCC are randomly joined or incorporated into chromosomes of cell hybrids, manifesting themselves as multiple unbalanced translocations. These three mechanisms are not mutually exclusive as they are independent and thus can all contribute to chromosomal instability following cell fusion. The overload and the centrosome mechanisms can be triggered by other processes leading to tetraploidy, even though cell fusion has more capabilities to provide a cell with an abnormal number of chromosomes; the cell-cycle conflict mechanism is specific for cell fusion.

this resistance occurs because normal cells provide an inhibitor, perhaps repressed in the transformed cells, which interrupts the pathway that links chemotherapy with apoptosis. Considering that fusion of tumorigenic cells with normal cells produces hybrids that are non-tumorigenic until they lose some of the chromosomes of the normal cells — the observation that led to the concept of tumour suppressors[109] — we speculate that cancerous cells can escape therapy by fusing to normal cells. Most of the resulting hybrids will be killed by chromosomal instability, but survival of just a few might be sufficient for a tumour to recur. It is also possible that tumour cells can escape treatment by fusing to each other, thereby complementing their individual drug-resistance capabilities[110].

The suppression hypothesis. Although we focused on potentially carcinogenic properties of cell fusion, the observations that fusion to normal cells inactivates the ability of cancer cells to make tumours[109] and the findings that most hybrids are not viable suggests that viruses can also kill tumour cells by fusing them to normal cells (FIG. 2f).

The reactivation hypothesis. This idea is based on reports that some viruses, such as SV40, can be reactivated following cell fusion[22] (FIG. 2g). Therefore, one can surmise that a non-oncogenic virus can cause fusion and thus result in expression of a silenced oncogenic virus.

Unrelated findings. The final conjecture is whether our observations are related to cancer development at all. Indeed, the consequences of cell fusion in the dish and the body could, for some reason, be different or the incidence of accidental cell fusion in the body could be negligible. What the incidence is and what it should be to be relevant for cancer development is unclear, especially considering that the incidence of cancer is astronomically small relative to the number of cells born in the body over the decades required to develop a tumour.

Does the viral fusion–cancer link exist?

The notion that cell fusion contributes to cancer implies that at least some cells in some cancers are hybrids. Therefore, testing this idea will require finding whether these hybrids exist.

Cell hybrids can be unambiguously detected in chimeric animals[68,111], which are made of two or more genetically distinct cell populations — finding genetic markers from more than one population in a cell would indicate this cell as a hybrid, assuming that possible artefacts are excluded. Therefore, one approach to test whether cell fusion has any role in cancer development is to analyse cancers that arise in chimeras. Of particular interest would be mouse cancer models that produce aneuploid cancers in the triploid to tetraploid range.

Learning whether cell hybrids are ever formed during the development of human cancer is more desirable because they are of clinical interest, and because mechanisms of human and mouse carcinogenesis differ in many aspects[112], including the latency of viral-induced cancers, which in humans can be as long as 20 mouse lifetimes. However, detecting cell fusion and its consequences in humans is also more difficult because hybrids of genetically identical cells have no known hallmarks. In principle, karyotypic diversity of cancer cells provides an opportunity to identify hybrids that might form during tumour progression, but chromosomal instability of hybrids can make this approach difficult.

Glossary

Aneuploidy
Any deviation from the exact multiple of the euploid number of chromosomes for the species. This includes a deviation in the number of whole chromosomes (numerical aneuploidy) and in parts of the chromosomes (segmental aneuploidy).

Cell hybrids
Mononuclear cells produced by mitosis of heterokaryons. The best-known example of cell hybrids are hybridomas.

Chromosomal instability
(CIN). An abnormally high frequency of chromosomal aberrations in a cell or cell population, such as chromosome losses, gains or translocations. Chromosomal instability leads to aneuploidy.

Heterokaryon
Multinuclear cells produced by fusion of different cells.

Osteoclasts
Syncytia whose function is to dissolve bone.

Syncytium
A cell produced by fusion that has more than a few nuclei.

A largely unexplored approach is to analyse cancer cells of patients who received bone-marrow or organ transplants and, therefore, are made of two genetically distinct cell populations. Hybrids between cells of these populations can be identified unambiguously by microsatellite analysis of individual cancer cells. If the transplant was from a person of the opposite sex, sex chromosomes could also be used as markers to identify cell hybrids[113,114], which should have one Y and three X chromosomes immediately after fusion, but this assay might be unreliable because of chromosomal instability.

One approach that could be applied to any patient is to analyse binuclear or multinuclear cells often found in neoplasia (FIG. 3). For example, the fraction of binuclear cells increases with the progression from normal cells to cervical cancer[115]. Binuclear and multinuclear cells can be relatively easily identified and, importantly, can be made by two events only: failed cytokinesis or cell fusion. Which of these has taken place could be determined through whether the nuclei of a cell are synchronized in the cell cycle (D.D. & Y.L., unpublished data). A failure to divide produces a binuclear cell whose nuclei are both in the G1 phase of the cell cycle and then progress to mitosis synchronously or are arrested, again synchronously, at a checkpoint. However, a binuclear cell produced by fusion can have asynchronous nuclei if the parental cells happened to be asynchronous at the time of fusion. These nuclei may eventually synchronize, but for some time they would have a different amount of DNA that can be identified by cytometry, or differ in other markers of the cell cycle that cannot be changed rapidly following cell fusion and can be detected in human cells.

Analysing binuclear or multinuclear cells, rather than mononuclear cancer cells, in metastatic lesions might also help to determine whether cell fusion contributes to metastasis, as shown by the finding of myeloma nuclei in osteoclasts from myeloma patients[47]. This might explain the propensity of myeloma metastases to dissolve bone. A similar approach systematically applied to binuclear or multinuclear cells in metastasis of other cancers would reveal how frequent cell fusion is in these lesions. Cases in which metastases differ genetically from the primary tumours[106] would be of particular interest. Although multinuclear cells are easier to identify, analysing cells that contain only two or three nuclei might be more informative, as they are likely to be more frequent and also more likely to produce proliferating progeny and thus contribute to cancer development[22].

More insights into properties of hybrids and, therefore, new tools to detect them in cancer might come from determining how cell fusion causes CIN.

How does cell fusion cause CIN?

What causes CIN during cancer development is not entirely clear. It is well documented that the maintenance of CIN is enabled by deficiencies in proteins that police genome integrity, such as p53 (REFS 27,116), but the cause(s) of CIN in sporadic cancers remains uncertain[117–120]. The primary suspects are mutations that cause deregulation of telomere maintenance[121] or affect mitotic fidelity[119]. However, it remains unclear why microsatellite instability, which increases the mutation rate by several orders of magnitude in some diploid cancers, fails to induce CIN[122]. In addition, why would acquiring a mutation that adversely affects every subsequent mitosis provide a cell with the proliferation advantage required to form a tumour, and why have mutations that cause chromosomal instability not been identified in the majority of sporadic cancers[119]? These questions can be answered if CIN is caused by a transient event, such as cell fusion, that does not permanently affect mechanisms required for proliferation.

In principle, cell fusion can cause chromosomal instability, thus producing cells with abnormal sets of chromosomes, by three mechanisms that are not necessarily mutually exclusive. One, which we call the overload hypothesis (FIG. 4a), proposes that tetraploidy, a natural consequence of cell fusion, is sufficient to trigger CIN because the increased number of chromosomes overwhelms the mitotic machinery, thus making abnormal chromosome segregation more likely[25–27,123]. Because many cell lines and even some mammals are tetraploid but have a stable genome[124,125], it is possible that the overload caused by tetraploidy might lead to CIN only if the mitotic machinery is already deregulated, for example owing to oncogene expression[126].

The centrosome hypothesis (FIG. 4b) is based on the fact that cell fusion results in cells with an abnormal number of centrosomes, because all centrosomes that are present in the parental cells at the time of fusion are combined. Two centrosomes are normally present in a mitotic cell and these form a bipolar mitotic spindle to segregate chromosomes between the two daughter cells during mitosis. Therefore, any excess in the number of centrosomes might result in tripolar and tetrapolar, rather than bipolar, mitoses. After cell fusion, a tripolar mitosis would divide eight sets of chromosomes among three cells, which must produce aneuploid cells, whereas a tetrapolar mitosis can, in principle, produce four cells with a nearly normal number of chromosomes.

The third possibility, the conflict hypothesis, suggests that CIN results from the fundamental property of cell fusion to combine different cells (FIG. 4c). The properties of these cells need to be reconciled in the hybrids in ways that are largely unknown, but clearly are not always amicable. One conflict is PCC, which is likely to break chromosomes, especially if they are forced to condense during DNA replication, a process that involves numerous breaks in the DNA. The DNA fragments produced by PCC are known to be randomly incorporated into the chromosomes of daughter cells[127,128], which might explain massive unbalanced chromosomal translocations of the hybrids that we[57] and others[23] have observed. Indeed, this was proposed as a cause of chromosomal aberrations in cells fused by viruses[127,128]. The conflict hypothesis is also consistent with the ability of hybrids to retain as much as the whole genome of each parent or as little as only a few megabases of DNA from one of them, a property exploited for genome mapping[129]. Testing whether chromosomal instability triggered by cell fusion has any hallmarks of its origin might provide clues to identifying hybrids in cancer.

Conclusions

Overall, the incidence of accidental cell fusion caused by viruses or other agents in normal or neoplastic tissues is unknown and the consequences of this fusion are poorly understood. However, the potential pathogenic effects of accidental cell fusion suggest that assuming that this event is harmless is unreasonable. Some of the tools and approaches that are required to test whether cell fusion has any role in cancer are available, whereas others are yet to be developed. Overall, testing the hypotheses outlined above will require collective effort, which we hope this Perspective will encourage.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
E1A | ERVWE1 | p53

SUPPLEMENTARY INFORMATION

See online article: S1 (table)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

Family Subfamily	Genera with evidence of human (cell) infection	Evidence of human as host (examples)	Evidence for cell-cell fusion (examples)	Example of viruses known to cause a human disease	Example of viruses with no association with disease, reported non-pathogenic, with benign pathogenicity, under-studied, or infection of human cells underappreciated.
Arenaviridae	*Arenavirus*	Mopeia, Mobala, IPPY, Junin, Lassa Viruses [1]	Junin Virus [2-5]	Lassa virus-hemorrhagic fever LCMV-menengitis, encephalitis [6-8]	Mopeia, Ippy, Mobola and other viruses- no association with disease in animals or cultured cells. [1]
Bornaviridae	*Bornavirus*	Human Bornavirus [9]	Borna Disease Virus, [10, 11]	Brona Disease Virus-Obesity?[12] Autism/other behavioral disorders?[13]	Borna Disease Virus-some asymptomatic [14]
Bunyaviridae	Orthobunyavirus Hantavirus Nairovirus [15] Phlebovirus [16] Tospovirus (laboratory [17])	Hantaviruses [18, 19]	La Crosse Bunyavirus, Hemorrhagic fever with renal syndrome (HFRS), Hantaan Virus, [20-23]	Hantaviruses [18, 19]	Toscana virus [24] Andes Hantavirus [25] Bwamba Virus [26] La Crosse Virus [27] Puumala Virus [28]
Coronaviridae	Coronavirus Torovirus	Restpiratory tract bovine cornoaviruses [29]	Restpiratory tract bovine cornoaviruses, SARS coronavirus, toroviruses [29-31]	SARS[32]	common human coronaviruses 229E and OC43, common cold [32] HCoV-NL63 (usually subclinical symptoms)[33] Others [34]
Filoviridae	Marburgvirus Ebolavirus	Ebola and Marburg Viruses [35]	Ebola and Marburg Viruses [36, 37]	Ebola virus-Hemorrhagic fever[35]	*NONE, except Ebola virus-Asymptomatic in few hosts* [38]
Flaviviridae	Flavivirus Pestivirus Hepacivirus	Hepatitis C virus, [39-41]	Hepatitis C virus [42-44]	Hepatitis C virus [39-41] West Nile Virus[45]	GB virus type C/hepatitis G virus [45-47]
Hepadnaviridae	Orthohepadnavirus	HBV [48]	Hepatitis B virus [49]	HBV [48]	HBV has a range of clinical symptoms, from benign to shortened life expectancy [50]

Herpesviridae	Alphaherpesvirinae	Varicella-Zoster Virus [51] Herpes Simplex [52]	Herpes Simplex Virus [53-55] Varicella Zoster [56]	Varicella-Zoster Virus [51] Herpes Simplex [52]	HSV-1 and HSV-2 benign to asceptic meningitis [57] VZV usually benign and self-limitted [57] Bovine mammillitis virus can be grown in human cells [58] zoonoses?!
	Betaherpesvirinae	Cytomegalovirus [59]	Cytomegalovirus [60]	Cytomegalovirus [59]	HHV-6 infects almost all children [61] giving rise to minor symptoms [62], can be a problem in transplant recipients and otherwise immune-compormised hosts [63]. HHV-7, can be a problem in pediatric stem cell transplants [64]. However whether there is a a cause-effect relationship is far from understood [65]
	Gammaherpesvirinae	Epstein-Barr Virus (HHV-4) [66] Kaposi Sarcoma (HHV-8) [67]	Epstein-Barr [68]	Epstein-Barr Virus [66] Kaposi Sarcoma [67]	Zoonotic Kaposi-like virus from Gorilla GorRHV1, exists, clinical relevance unknown [69]?
Iridoviridae	Iridovirus [70] Ranavirus	Frog Virus 3, Bullfrog Edema Virus and Lucke titurus virus can infect and replicate in human cells in culture [70]	[71]	No evidence of naturally occuring human infection	No evidence of naturally occuring human infection
Orthomyxoviridae	Influenzavirus A Influenzavirus B Influenzavirus C Thogotovirus	Influenzavirus A [72]	Parainfluenza virus, influenza virus [42, 73, 74]	Influenzavirus A [72]	Influenza C seemingly benign [75] Thogotovirus infection of humans may be limited by human protein [76]
Paramyxoviridae /Paramyxovirinae	Respirovirus Morbillivirus Henipavirus Avulavirus [77] Rubulavirus	Hendra/Nipha Virus [78] Morbillivirus [48]	Hendra Virus, Nipah Virus, Measles Virus [79, 80] Sendai Virus	Hendra/Nipha Virus [78] Morbillivirus [48]	some Morbilliviruses have weak or no links to human disease [48] HPIV-4 has mild respiratory, or asymptomatic infections [81]
/Pneumovirinae	Pneumovirus Metapneumovirus	HRSV cause of bronchiolitis and pneumonia	HRSV [83]	HRSV cause of bronchiolitis and pneumonia [82]	Metapneumovirus (hMPV) elicit some respiratory tract infections in children [84]

		[82]			
Parvoviridae /Parvovirinae	Erythrovirus Dependovirus "BPV-like viruses" (can agglutinate human cells [85])	B19 [86]	B19 may be fusogenic [87]	B19 [86]	AAVs are considered benign and exploited for gene therapy [88]
Poxviridae Chordopoxvirinae	Chordopoxvirinae Orthopoxvirus [89] Parapoxvirus Suipoxvirus (laboratory setting [90]) Molluscipoxvirus [91] Yatapoxvirus [92] Entomopoxvirinae Betaentomopoxvirus (nonproductively or artificially [93])	Variola, Smallpox [89]	Variola Virus and others [94]	Variola Virus Smallpox [89]	Both ORFV and BPSV cause occupational infections in humans with lesions characterized by large, painful nodules in the hands and, less frequently, the face [95] Yaba Monkey Tumor virus (can transform human cells in culture, not much studied [92]), swine-pox virus [90] Amsacta moorei (AmEPV), transient infection [93] Molluscum Contagiosum Virus, self-limiting infections, benign [91]
Reoviridae	Orthoreovirus Obrivirus [104] Rotavirus Coltivirus [96] Seadornavirus [96] Aquareovirus [97]	Coltivirus [96] Seadornavirus [96]	Nelson Bay Virus, Baboon Reovirus, Avian Reovirus [98-101]	Coltivirus [96] Seadornavirus [96]	Reoviruses considered benign and used in therapy [102] although can induce apoptosis in heart and neurons [103] Infection and replication of some aquareoviruses are not restricted to fish [97] Blue Tong Virus can infect and induce apoptosis in human cells [104]
Retroviridae	Retroviridae Orthoretrovirinae Alpharetrovirus Betaretrovirus Gammaretrovirus Deltaretrovirus	HIV [105]	Human endogenouse viruses [106]	HIV [105]	MPMV [107], Simian Foamy Virus [108], Multiple-Sclerosis Associated Virus [109], Endogenous Retrovirus K [110] etc. etc…

	Epsilonretrovirus Lentivirus Spumaretrovirinae Spumavirus				
Rhabdoviridae	Vesiculovirus [111] Lyssavirus [112]	VSV[111], Rabies	Cocal virus, VSV [113, 114]	Lyssavirus (Rabies) Virus [112]	VSV harmless to humans [111], vesicular stromatis in lifestock [115]
Togaviridae	Alphavirus Rubivirus	Eastern, Western, Japanese, St. Louis & Venezuelan Equine Encephalitis Viruses, West Nile Virus, Yellow Fever Viruses. Rift Valley Virus, Dengue Virus [116]	Sindbis Virus, [117, 118]	Rubella-Congenital Rubella Syndrome [119] Sindbisvirus-arthritis[120] Ross River Virus-arthritis[121]	some strains of Semilsky Forest Virus, Sgyiama Virus and others [122-124]

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